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(54) Modified hIL-6.

(57) Provided are PEGylated "Interleukin-6" derivatives (PEG-IL-6) having an extended plasma half-life, as well as enhanced *in vivo* IL-6 biological activities.
Methods for producing the modified glycosylated and unglycosylated IL-6 proteins or polypeptides, as well as, for their use in treating hematopoietic disorders and difficiencies, particularly acute thrombocytopenia, are also provided.

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MODIFIED hIL-6

FIELD OF THE INVENTION

The present invention relates to modified glycosylated and unglycosylated proteins and polypeptides possessing interleukin-6 (hereafter referred to as IL-6) activities where the modification comprises chemical modification of at least one amino group or carboxyl group on the proteins and polypeptides. The invention also relates to a process for producing the modified proteins or polypeptides, and to their use in treating haematopoietic disorders and deficiencies, particularly acute thrombocytopenia.

BACKGROUND OF THE INVENTION

Interleukin-6 is a multifunctional cytokine (Kishimoto, T. and T. Hirano, *Ann. Rev. Immunol.*, 6 :485 (1988)). Included among its diverse biological activities are the induction of the terminal differentiation of B cells to plasma cells, the differentiation of killer T cells and nerve cells, as well as the acute phase protein synthesis of hepatocytes. It also stimulates the growth of hybridoma/plasmacytoma/myeloma cells, T cells, and hematopoietic stem cells. Differentiation inducing activity on megakaryocytes, leading to the production of platelets, has also been reported recently (Ishibashi, T., *et al., Blood* 74 :1241, (1989)).

One example of glycosylated proteins or polypeptides possessing interleukin-6 activities is human interleukin-6 (hereafter referred to as hIL-6). There are a number of reports on processes for producing hIL-6 ; for example, production by human T cell hybridoma cells (Okada, M., *et al., J. Exp. Med.*, 157 :583 (1983)) or by human T cells transformed with human T cell leukemia virus (Japanese patent application, KOKAI NO. 61-115024). Human IL-6 may also be produced by recombinant DNA technologies which comprise transforming mammalian or bacterial cells with a vector carrying a DNA sequence encoding hIL-6 and then culturing these cells to obtain recombinant hIL-6. The resultant hIL-6 is a glycosylated protein if produced by mammalian cells, and an unglycosylated polypeptide if produced by bacterial cells. Both forms have been demonstrated to have interleukin-6 activities (EP 257406 ; WO 88/00206).

The mature fully functional hIL-6 polypeptide has 184 amino acid residues as predicted from the nucleotide sequence of its cDNA. However, polypeptides with one or more additional amino acid residues or (at most) 27 amino acid deletions at their N-terminus, as well as polypeptides with at most 50 amino acid deletions (or substitutions) at their C-terminus, are known to retain IL-6 activity (EP 257406 ; WO 88/00206 ; EP 363083 ; Brakenhoff, J.P.J., *J. Immunol.*, 143 :1175 (1989)).

Several methods have been used to try to prolong the plasma half-life of certain intravenously administered high molecular weight polypeptides. These include modification of the polypeptide with polyethylene glycol (PEG), dextran, poly[Glu-Lys], pullulan, modified polyaspartate, or fatty acids, as well as coupling with γ-globulin. The chemical modification with PEG (hereinafter referred to as PEGylation) of a few non-human derived enzymes, such as asparaginase, superoxide dismutase, or uricase, resulted in increased plasma half-life. However, a number of problems have been observed with PEGylation. Acylation of tyrosine residues on the protein can result in a lowering of the biological activity of the protein ; certain PEG-protein conjugates are insufficiently stable and therefore find no pharmacological use ; certain reagents used for PEGylation are insufficiently reactive and therefore require long reaction times during which protein denaturation and/or inactivation can occur. Also, the PEGylating agent may be insufficiently selective. Difficulties can also arise as a result of the hydrophobicity of the protein to be PEGylated ; in an aqueous medium hydrophobic proteins resist PEGylation at physiological pH. The criteria for effective PEGylation include not only whether the conjugated molecule has a combination of increased serum half-life and decreased immunogenicity, but also whether it is in fact a more potent pharmacological agent than its unmodified parent molecule. Given the broad range of differences in the physical characteristics and pharmacokinetics among proteins, it is impossible to predict in advance whether a protein can be successfully PEGylated and/or whether the PEGylated protein will still retain its biological activity without inducing untoward immunological responses.

For example, in WO87/00056, relating to the solubilization of proteins for pharmaceutical compositions using polym r conjugation, the adverse effect of PEGylation on the *in vitro* activity of IL-2 is described in Exampl IIB (Table I, page 20). Example IC (pag 19) references the IL-2 cell proliferation bioassay used. The results demonstrate that as more amino groups of the IL-2 are substituted with PEG, th PEGylated IL-2 undergoes a nearly 10-fold decreas in activity as compar d to th activity of unmodified IL-2.

Th covalent modification of lysine residues causes a r duction in bioactivity of certain proteins. Lysine modification with activated PEG-esters is random, difficult to control, and often results in reduced bioactivity of the modified protein (Goodson, R., *et al., Bio/Technology*, 8 :343 (April 1990)).

U.S. Pat nt No. 4,904,584 (February 27, 1990) describes a process for preparing PEGylated polypeptides. However, th process requires a pre-modification of th polypeptides by first preparing LDVs (lysine depleted variants) to obtain a polypeptide having a "suitable" number of reactive lysine residues. No evidence is present d that PEGylated derivativ s were actually obtained ; nor is their any vidence that these proposed modified polypeptides retained any biological activity. Further, there is no exemplification of the production of PEG-IL-6 nor any exemplification of retained activity.

As the *in vivo* half-life of IL-6 in blood is very short (Castell, J.V. *et al., Eur. J. Biochem.*, 177 :357 (1988)), it is desirable to increase hIL-6 plasma half-life and to thereby improve the pharmacokinetics and therapeutic efficacy of IL-6. To date, however, no one has been successful in so doing.

SUMMARY OF THE INVENTION

The present invention provides chemically modified glycosylated and unglycosylated proteins or polypeptides possessing a prolonged plasma half-life and improved *in vivo* IL-6 activities, particularly platelet producing activity, in primates, and especially in humans.

According to the invention, IL-6 is derivatized with polyethylene glycol (PEG) to produce PEG-IL-6 having prolonged plasma half-life and enhanced *in vivo* IL-6 biological activity. The IL-6 may be either glycosylated IL-6, unglycosylated IL-6, or biologically active fragments of either glycosylated or unglycosylated IL-6. Preferred amino acid sequences for IL-6 are set forth in the Sequence Listing and identified as Sequence Id Nos. 1, 2, and 3. The IL-6 can be either naturally or synthetically produced, either by recombinant methods or other methods known to those skilled in the art including chemical syntheses. Naturally produced IL-6 can be the expression product of a prokaryotic or eukaryotic host cell transformed or transfected with a DNA sequence encoding IL-6 or encoding a biologically active IL-6 fragment. A preferred host cell includes *E. coli*. The activated PEG used to produce PEG-IL-6 can be any of many activated PEG known to those skilled in the art including activated polyethylene glycol (4500), activated polyethylene glycol (5000), activated polyethylene glycol (12000), and the like. The particular activated PEG chosen can be any of a broad range of molecular weights as known to those skilled in the art and as used herein includes the molecular weight range of 4,500 to 12,000. Particularly preferred activated PEG includes the succinimidyl succinate derivatives of PEG and the bis-PEG derivatives of cyanuric chloride. The PEG can be attached to the IL-6 via one or more amino groups or one or more carboxyl groups. The number of PEG moieties per IL-6 protein, polypeptide or fragment can vary as can the molecular weight of the PEG moiety. Generally, the higher the degree of PEGylation, i.e., the greater the number of PEG groups per protein molecule, the greater the *in vivo* biological activity of the IL-6 ; similarly, the higher the molecular weight of the PEG used to PEGylate, the fewer the number of PEG groups per protein molecule required for *in vivo* activity. Generally, at least two, and preferably more than five PEG moieties, should be attached per IL-6 protein polypeptide, or fragment. Preferred PEG-IL-6 molecules include : PEG1-IL-6 ; PEG2-IL-6 ; PEG12M-IL-6 ; PEG(4500)IL-6, including fractions Fr45-0, Fr45-1, Fr45-2, Fr45-3, and Fr45-4 ; PEG(10000)IL-6, including fractions Fr100-0, Fr100-1, Fr100-2, Fr100-3, Fr100-4, and Fr100-5 ; PEG(12000)IL-6, including fractions Fr120-1, Fr120-2, Fr120-3, Fr120-4, and Fr120-5 ; and PEG(12000)IL-6', including the fractions Fr120'-1, Fr120'-2, Fr120'-3, Fr120'-4, and Fr120'-5.

The present invention also provides for a method to produce such improved IL-6 comprising chemical modification of the polypeptide. The present invention further provides for the use of such improved IL-6 for treating disorders associated with thrombocytopenia and/or impaired platelet functions. In cancer therapy, a dramatic drop in platelet count is frequently induced by the highdose administrations of carcinostatics ; this drop causes a variety of disorders such as excessive hemorrhaging. The improved IL-6 of the present invention is expected to be useful in reducing side effects associated with cancer chemotherapy. According to the method of the invention, haematopoietic disorders can be treated in an organism by administering a therapeutically effective amount of a composition comprising PEG-IL-6. The method further comprises the step of promoting platelet production. Also provided by the invention are pharmaceutical compositions for use in treatment of haematopoietic disorders in an organism ; the composition comprises a pharmaceutically acceptable solvent, diluent, adjuvant or carrier and, as the active ingredient, from about 0.0001 mg to about 10 mg per kg of body weight of the afflicted organism per day of PEG-IL-6, and preferably in the range of 0.004-1 mg per kg body weight per day. Also provided is a method for treatment of haematopoietic disorders in an organism by administering the nov l pharmaceutical compositions of PEG-IL-6.

BRIEF EXPLANATION OF THE DRAWINGS

Figure 1 is a graph showing the ffect of the administration of modified (Fr45-2) and unmodified hIL-6 on the number of peripheral platelets present aft r X-ray irradiation of mice.

Figure 2 is a graph showing the effect of the administration of modified (Fr45-2) and unmodified hIL-6 on th number of peripheral platelets after chemical carcinostatic treatment of mice.

Figur 3 is a graph showing th effect of the administration of modified (Fr120-1 to 120-5) hIL-6 administration on the number of peripheral platelets after X-ray irradiation of mice.

Figure 4 is a graph showing the *in vivo* persistence of modified and unmodified hIL-6 in mouse blood.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof which includes numerous illustrative examples of the practice of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Described below is the chemical modification with PEG of at least one amino group of polypeptides possessing IL-6 activities. This modification results in an increase in *in vivo* half-life in blood and an increase of *in vivo* platelet producing activity as compared to that of unmodified IL-6. Thus, in one aspect of the present invention, modified glycosylated and unglycosylated proteins or polypeptides (preferably polypeptides) possessing improved interleukin-6 activities, particularly platelet producing activity, in primates, and especially in humans, are provided where the modification comprises the attachment of PEG to the polypeptides.

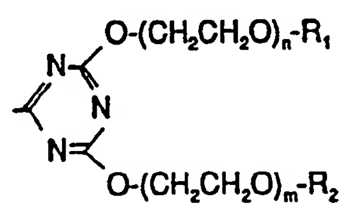
The attachment of PEG to the molecules possessing IL-6 activities may be done via the amino or carboxyl groups present in the polypeptides, and is preferably via the amino groups. For PEGylation via amino groups, at least one hydrogen atom of an amino group of the protein or protein fragment to be PEGylated is substituted with a group as shown in formula 1 or 2 :

FORMULA 1

$$-\overset{\displaystyle O}{\overset{\|}{C}}-CH_2CH_2-\overset{\displaystyle O}{\overset{\|}{C}}-O-(CH_2CH_2O)_n-R_1$$

n : a positive integer between 7 and 600.
R_1 : an alkyl group with one to three carbons.

FORMULA 2



n,m : a positive integer between 7 and 600, and may be equal or different.
R_1,R_2 :an alkyl group with one to three carbons, and may be the same or different.
In the present invention, hIL-6 polypeptides having substantially the following amino acid sequence are preferred as either the glycosylated or unglycosylated proteins or polypeptides possessing IL-6 activities :

SEQUENCE ID NO. : 1

ALA PRO VAL PRO PRO GLY GLU ASP SER LYS ASP VAL ALA ALA PRO
HIS ARG GLN PRO LEU THR SER SER GLU ARG ILE ASP LYS GLN ILE
ARG TYR ILE LEU ASP GLY ILE SER ALA LEU ARG LYS GLU THR CYS
ASN LYS SER ASN MET CYS GLU SER SER LYS GLU ALA LEU ALA GLU
ASN ASN LEU ASN LEU PRO LYS MET ALA GLU LYS ASP GLY CYS PHE
GLN SER GLY PHE ASN GLU GLU THR CYS LEU VAL LYS ILE ILE THR
GLY LEU LEU GLU PHE GLU VAL TYR LEU GLU TYR LEU GLN ASN ARG
PHE GLU SER SER GLU GLU GLN ALA ARG ALA VAL GLN MET SER THR
LYS VAL LEU ILE GLN PHE LEU GLN LYS LYS ALA LYS ASN LEU ASP
ALA ILE THR THR PRO ASP PRO THR THR ASN ALA SER LEU LEU THR
LYS LEU GLN ALA GLN ASN GLN TRP LEU GLN ASP MET THR THR HIS
LEU ILE LEU ARG SER PHE LYS GLU PHE LEU GLN SER SER LEU ARG
ALA LEU ARG GLN MET

As used herein "substantially" means that the polypeptides may have one or more amino acid modification(s) (deletions, additions, insertions, or substitutions) of the above amino acid sequence as long as the modification(s) does not have any adverse affect on the function and biological activity of the polypeptides. Examples of such modifications are described in published patent applications EP 257406, WO 88/00206 and EP 363083 as well as in Brakenhoff, J.P.J., (*J. Immunol.*, 143 :1175 (1989)).

The polypeptides may be produced by a number of methods including genetic engineering ; hIL-6 polypeptides produced by recombinant *E. coli* are preferred as they can be obtained in good purity and in large quantity. A polypeptide with exactly the above amino acid sequence or the above amino acid sequence with one methionine or a Met-Lys dipeptide added at the N-terminus is especially preferred. These hIL-6 polypeptides may be produced by the procedure disclosed in PCT patent application WO 88/00206 (Genetics Institute Incorporated). They may also be produced by first chemically synthesizing a DNA sequence encoding hIL-6 polypeptides (Haegeman, G., *et al., Eur. J. Biochem.*, 159 :625 (1986)) and then expressing the DNA in *E. coli* by the method of Souza *et al.* (WO 87/01132) (U.S. Patent No. 4,810,643).

As used herein in the formulae, m and n indicate average values ; m and n may be equal or different, are preferably equal and between 7 and 600, more preferably between 7 and 250, and most preferably between 30 and 150. The average molecular weight of the PEG used in the present invention may be between 300 and 30,000 ; and is preferably between 1,000 and 20,000 ; and is most preferably 12,000. The protecting groups for the hydroxyl group of the PEG, indicated as R_1 and R_2, may be alkyl groups, with one to three carbon atoms, such as methyl, ethyl, *n*-propyl or *i*-propyl groups, and are preferably methyl groups.

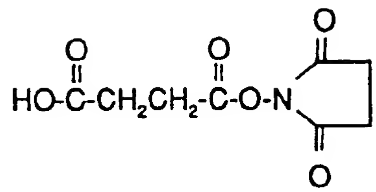
As a further aspect of the present invention there are provided processes for producing the PEGylated glycosylated proteins or polypeptides possessing IL-6 activities. The PEGylation of the amino groups on polypeptides possessing IL-6 activities (hereafter referred to as IL-6 polypeptides) may be achieved via succinimide (formula 1) or triazine (formula 2), and is preferably via succinimide. In the PEGylation via succinimide, PEG shown in formula 3 is coupled with the compound shown in formula 4 to obtain the compound shown in formula 5, which is then coupled to IL-6 polypeptides. Some of the activated PEGs, represented by the generic formula 5, are commercially available from, for example, Nippon Oil & Fats Co. (Tokyo, Japan). Formula 5 is a generic formula representative of succinimidyl succinate derivatives of PEG.

FORMULA 3 : PEG

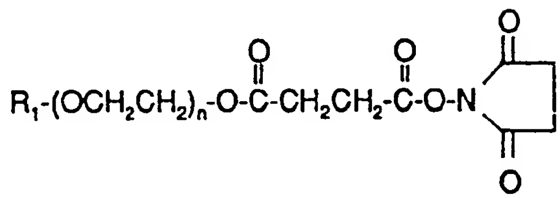
$HO\text{-}(CH_2CH_2O)_n\text{-}R_1$

n, R_1 : same as in formula 1.

FORMULA 4



FORMULA 5 : succinimidyl succinate derivative of PEG

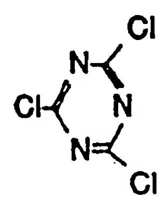


n,R_1 : same as in formula 1.

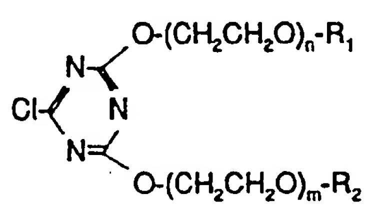
The coupling of activated PEG (formula 5) to IL-6 polypeptides may be achieved by incubating in 0.25 M sodium borate buffer (pH 8.0-8.5) for one to three hours at 4°C. The activated PEG may be added to the reaction mixture serially in small quantities to avoid its degradation. After the reaction, the PEGylated IL-6 polypeptides can be separated from unreacted materials by gel filtration and ionexchange column chromatography.

For PEGylation via triazine, PEG shown in formula 3 is coupled with the compound shown in formula 6 to obtain the compound shown in formula 7 ; the formula 7 activated PEG is then coupled to IL-6 polypeptides. Some of the compounds represented by the generic formula 7 are commercially available from, for example, Seikagaku Kogyo Co. (Tokyo, Japan).

FORMULA 6



FORMULA 7 : bis-PEG derivative of cyanuric chloride.



n,m,R_1,R_2 : same as in formula 2.

The coupling of activated PEG (formula 7) and IL-6 polypeptid s may be achieved by incubating in 0.25 M sodium borat buffer (pH 10.0) for two to twenty hours at 4°C to room t mp rature. The activated PEG may be added to the reaction mixture serially in small quantities to avoid its degradation. After the reaction, the PEGylated IL-6 polypeptides can be separated from unreacted materials by gel filtration and ion-exchange column chromatography.

The PEGylation of the carboxyl groups on IL-6 polypeptides may b achiev d by coupling the polypeptides

with the PEG shown in formula 8.

FORMULA 8

$H_2NCH_2CH_2CH_2O\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2CH_2NH_2$

n : same as in formula 1.

As a general rule, the higher the degree of PEGylation, i.e., the greater the number of PEG groups per protein molecule, the better the *in vivo* activity ; and, the higher the molecular weight of the PEG used to PEGylate, the fewer the number of PEG groups per protein molecule required for *in vivo* activity. Generally, at least two and preferably more than five PEG moieties should be attached per IL-6 protein.

As used herein, the following abbreviations and terms include, but are not necessarily limited to, the following definitions.

Activated PEG1 is a generic term used to indicate activated polyethylene glycol (4500) ;

Activated PEG2 is a generic term used to indicate activated polyethylene glycol (5000) ;

Activated PEG12M is a generic term used to indicate activated polyethylene glycol (12000) ;

PEG(4500)IL-6 is a generic term used to indicate the product formed upon the reaction of the succinimidyl succinate derivatives of PEG(4500) with IL-6 ;

PEG(10000)IL-6 is a generic term used to indicate the product formed upon the reaction of the bis-PEG (5000) derivatives of cyanuric chloride with IL-6 ; and

PEG(12000)IL-6 is a generic term used to indicate the product formed upon the reaction of the succinimidyl succinate derivatives of PEG(12000) with IL-6.

The numbers in parentheses, i.e., 4500, 5000, and 12000 refer to the average molecular weight of polyethylene glycol.

As used herein, "haematopoietic disorders and deficiencies" include but are not limited to thrombocytopenia, granulocytopenia, and anemia.

The PEGylated IL-6 of the present invention has a much longer plasma half-life and upon administration to mice has a far superior capacity to increase the number of platelets compared to unmodified IL-6 polypeptides or glycosylated IL-6 ; further, the PEGylated IL-6 of the invention has very low toxicity. Thus, in another aspect of the present invention, therapeutic agents for treating haematopoietic disorders, particularly acute thrombocytopenia, and deficiencies are provided. Such agents comprise a therapeutically effective amount of the PEGylated IL-6 of the present invention in an admixture with a pharmaceutically acceptable carrier. The agents may be administered orally as tablets or capsules, or parenterally by injection. Generally, the daily dosage regimen is in the range of 0.0001 -10 mg/kg weight (as polypeptide), and preferably in the range of 0.004-1 mg/kg.

The following examples illustrate practice of the invention.

Example 1 relates to the preparation of Fr45-0 from hIL-6 polypeptide and an activated PEG1 (methoxypolyethylene glycol (4500) succinimidyl succinate).

Example 2 relates to the SDS-PAGE characterization of Fr45-0.

Example 3 relates to the preparation of Fr100-0 from hIL-6 polypeptide and an activated PEG2 (bis-methoxypolyethylene glycol (5000) cyanuric chloride).

Example 4 relates to the preparation of four fractions of PEGylated hIL-6 polypeptide : Fr45-1, Fr45-2, Fr45-3, and Fr45-4 from hIL-6 and the activated PEG1 of Example 1.

Example 5 relates to the SDS-PAGE characterization of the four fractions of Example 4.

Example 6 demonstrates the capacity of the four fractions obtained in Example 4 capacity to induce IgM production in a B cell leukemic cell-line.

Example 7 compares the *in vivo* platelet producing activity of hIL-6, Fr45-0 and Fr100-0 (as prepared in Examples 1 and 3).

Example 8 compares the *in vivo* platelet producing activity of hIL-6 and of the four fractions of Example 4.

Example 9 relates to the subcutaneous administration of hIL-6 or PEGylated hIL-6(Fr45-2, produced in Example 4) to acute-thrombocytopenic mice following X-ray irradiation.

Example 10 relates to the subcutaneous administration of hIL-6, or PEGylated hIL-6 (Fr45-2, produced in Example 4) to acute-thrombocytopenic mice following treatment with the chemical carcinostatic cyclophosphamide.

Example 11 relates to th preparation of five fractions of PEGylat d hIL-6 polypeptide, Fr100-1, Fr100-2, Fr100-3, Fr100-4, and Fr100-5, from hIL-6 and the activat d PEG2 of Example 3.

Example 12 relat s to the coupling of hIL-6 polypeptide to an activated PEG12M (methoxypolyethylene glycol (12000) succinimidyl succinate) and the preparation therefrom of five fractions of PEGylated hIL-6 polypeptide : Fr120-1, Fr120-2, Fr120-3, Fr120-4, and Fr120-5.

Example 13 provides the results of a study to compare the level of platelet production following the admini-

stration of the hIL-6 (produced in Example 4) or its PEGylated derivatives (produced in Examples 11 and 12).

Example 14 relates to the subcutaneous administration of PEGylated hIL-6 (produced in Exampl 12 : Fr120-1 to Fr120-5) to acute-thrombocytopenic mice following X-ray irradiation.

Example 15 demonstrates th *in vivo* persistence of PEGylated hIL-6 (Fr45-2, Fr120-1, and Fr120-2 produced in Examples 4 and 12) as compared to unmodified hIL-6.

Example 16 describes the preparation of five fractions of PEGylated hIL-6 : Fr120'-1 to Fr120'-5 from the hIL-6 polypeptide of Example 4 (however, without the cathepsin C treatment) and the activated PEG12M of Example 12.

Example 17 provides the results of an acute toxicity assay of the hIL-6 of Example 4 and its PEGylated derivatives Fr45-2, Fr100-2, and Fr120-2 (produced in Examples 4, 11, and 12).

In Examples 7-10 and 13-17, the PEGylated hIL-6 polypeptides of the present invention were dissolved in PBS containing 0.1% normal mouse serum. The concentration of the solutions was adjusted so that the final volume administered was 100 µl.

EXAMPLE 1

A DNA sequence encoding the hIL-6 polypeptide with the following amino acid sequence was chemically synthesized and then introduced and expressed in *E. coli* as described by Souza *et al.* (WO 87/01132).

SEQUENCE ID NO. : 2

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MET ALA PRO VAL PRO PRO GLY GLU ASP SER LYS ASP VAL ALA ALA
PRO HIS ARG GLN PRO LEU THR SER SER GLU ARG ILE ASP LYS GLN
ILE ARG TYR ILE LEU ASP GLY ILE SER ALA LEU ARG LYS GLU THR
CYS ASN LYS SER ASN MET CYS GLU SER SER LYS GLU ALA LEU ALA
GLU ASN ASN LEU ASN LEU PRO LYS MET ALA GLU LYS ASP GLY CYS
PHE GLN SER GLY PHE ASN GLU GLU THR CYS LEU VAL LYS ILE ILE
THR GLY LEU LEU GLU PHE GLU VAL TYR LEU GLU TYR LEU GLN ASN
ARG PHE GLU SER SER GLU GLU GLN ALA ARG ALA VAL GLN MET SER
THR LYS VAL LEU ILE GLN PHE LEU GLN LYS LYS ALA LYS ASN LEU
ASP ALA ILE THR THR PRO ASP PRO THR THR ASN ALA SER LEU LEU
THR LYS LEU GLN ALA GLN ASN GLN TRP LEU GLN ASP MET THR THR
HIS LEU ILE LEU ARG SER PHE LYS GLU PHE LEU GLN SER SER LEU
ARG ALA LEU ARG GLN MET
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E. coli cells (300 g), that accumulated hIL-6 polypeptide, were harvested by centrifugation for 10 min at 3500*g*. The hIL-6 polypeptide was extracted, solubilized and refolded as described in EP 257406. Approximately 2.9 g of the hIL-6 polypeptide, which was a single band on SDS-PAGE and had the predicted molecular weight of 21K, was obtained.

An activated PEG1 (a succinimidyl succinate derivative of polyethylene glycol, which is methoxypolyethylene glycol succinate with an average molecular weight of 4,500 and coupled to N-hydroxysuccinimide) was commercially obtained from Nippon Oil & Fats Co. (Tokyo, Japan).

The hIL-6 polypeptide (200 µg) was incubated with 1.5 mg of the activated PEG1 in 370 µl of 0.25 M sodium borate buffer (pH 8.5) for two hours at 4°C. The reaction was stopped by lowering the pH by adding 2 N hydrochloric acid. The molar ratio of the activated PEG to the free amino groups on the IL-6 polypeptide was approximately two to one. The reaction mixture was applied to a gel filtration column equilibrated with phosphate buffered saline (PBS) to exchange the buffer and was then subjected to the separation procedure below.

Th buffer-exchanged s lution (3.5 ml) was appli d to an HPLC g l-filtration column filled with TSK-gel G3000SW (Toso Co., Tokyo, Japan). The PEGylated hIL-6 polypeptide, having one to three PEG moieties per molecul , was eluted in the first peak to give a yield of approximately 20 µg. The PEGylated hIL-6 polypeptide so obtained was termed Fr45-0.

EXAMPLE 2

The Fr45-0 obtained in Example 1 was characterized by SDS-PAGE (Fast System, Pharmacia ; 10-15% gradient gel). The polypeptides were stain d with silver staining and the molecular weights were stimated by comparison with standard molecular weight markers (Bio-Rad, Richmond, CA, USA). The apparent molecular weights of Fr45-0 were 23K, 37K, and 50K.

EXAMPLE 3

An activated PEG2 (an activated polyethylene glycol with an average molecular weight of 10,000 ; synthesized by coupling two polyethylene glycol (average molecular weight 5,000) monomethylether molecules with cyanuric chloride) was commercially obtained from Seikagaku Kogyo Co., Tokyo, Japan.

The hIL-6 polypeptide (200 µg) obtained in Example 1 was incubated with 3.5 mg of the activated PEG2 in 370 µl of 0.25 M sodium borate buffer (pH 10.0) for two hours at room temperature. The reaction was stopped by lowering the pH by adding 2 N hydrochloric acid. The molar ratio of the activated PEG to the free amino groups on the IL-6 polypeptide was approximately two to one. The reaction mixture was applied to a gel filtration column equilibrated with PBS to exchange the buffer and then subjected to the separation procedure described in Example 1. The PEGylated hIL-6 polypeptide with one to two PEG moieties per molecule was eluted in the first peak to give a yield of approximately 20 µg. The PEGylated hIL-6 polypeptide so obtained was termed Fr100-0.

The apparent molecular weights of Fr100-0 were determined to be 28K and 42K as described in Example 2.

EXAMPLE 4

A DNA sequence encoding the hIL-6 polypeptide with the following amino acid sequence was chemically synthesized and then introduced and expressed in *E. coli* as described by Souza *et al.* (WO 87/01132).

SEQUENCE ID NO. : 3

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MET LYS ALA PRO VAL PRO PRO GLY GLU ASP SER LYS ASP VAL ALA
ALA PRO HIS ARG GLN PRO LEU THR SER SER GLU ARG ILE ASP LYS
GLN ILE ARG TYR ILE LEU ASP GLY ILE SER ALA LEU ARG LYS GLU
THR CYS ASN LYS SER ASN MET CYS GLU SER SER LYS GLU ALA LEU
ALA GLU ASN ASN LEU ASN LEU PRO LYS MET ALA GLU LYS ASP GLY
CYS PHE GLN SER GLY PHE ASN GLU GLU THR CYS LEU VAL LYS ILE
ILE THR GLY LEU LEU GLU PHE GLU VAL TYR LEU GLU TYR LEU GLN
ASN ARG PHE GLU SER SER GLU GLU GLN ALA ARG ALA VAL GLN MET
SER THR LYS VAL LEU ILE GLN PHE LEU GLN LYS LYS ALA LYS ASN
LEU ASP ALA ILE THR THR PRO ASP PRO THR THR ASN ALA SER LEU
LEU THR LYS LEU GLN ALA GLN ASN GLN TRP LEU GLN ASP MET THR
THR HIS LEU ILE LEU ARG SER PHE LYS GLU PHE LEU GLN SER SER
LEU ARG ALA LEU ARG GLN MET
```

This amino acid sequence has N-terminal residues of Met-Lys-Ala-Pro- and thus can be conveniently converted to Ala-Pro-, the natural hIL-6 sequence, by cleaving off the Met-Lys using cathepsin C.

E. coli cells (300 g), that had accumulated hIL-6 polypeptide, were harvested by centrifugation for 10 min at 3500*g*, and the hIL-6 polypeptide was extracted, solubilized and refolded as described in EP 257406. After changing the buffer to 20 mM sodium acetate buffer, th polypeptide was treated with 6 U of cathepsin C (Boeringer Mannheim GmBH, Mannheim, Germany) for one hour at room temperature. The reaction mixture was quick-chilled and sodium ph sphat buffer (pH 6.0) was added to a final concentration of 2 mM. The mixture was applied to a hydroxyapatite column equilibrated with 2 mM sodium phosphate buffer (1300 mmho, pH 6.0), and the hIL-6 was eluted in a peak fraction of 1200 ml with the same buffer. This fraction was then applied to a CM-Sepharose column equilibrated with 20 mM sodium acetate buffer (pH 6.0). The column was washed

with the same buffer, and th hIL-6 was eluted in a peak fraction of 580 ml with a linear gradient of 0-0.3 M NaCl in the same buffer.

Approximately 1.5 g of th hIL-6 polypeptide, which was a single band on SDS-PAGE and had the predicted molecular weight of 21K, was obtained. The N-t rminal sequence was confirmed to be Ala-Pro-Val-Pro- by direct sequence analysis.

The hIL-6 polypeptide (100 mg) was dissolved in 100 ml of 0.1 M sodium borate buffer (pH 8.5) and 1125 mg of the activated PEG1 of Example 1 was added to the solution in an ice bath with stirring. The activated PEG1 was added either all at one time or in five additions at 30 min intervals, and the latter was found to give a better yield of the coupled product. Accordingly, the subsequent purification steps were carried out on the reaction mixture to which the activated PEG1 was added in five separate, sequential additions.

The reaction mixture was concentrated to 10 ml using YM10 ultrafiltration membrane (Amicon, Danvers, MA, USA) and was applied to a Sephadex G100 column equilibrated with 20 mM sodium acetate buffer (pH 6.0). The PEGylated hIL-6 polypeptide was eluted in four fractions (hereafter referred to as Fr45-1, Fr45-2, Fr45-3, Fr45-4) with the same acetate buffer. Upon SDS-PAGE analysis, each fraction gave a main band of 91K, 68K, 41K, and 26K ; respectively. The yield of each fraction was 2.9 mg, 4.0 mg, 2.9 mg, and 2.5 mg ; respectively.

EXAMPLE 5

The four fractions obtained in Example 4 were characterized by determining the number of free amino groups and by SDS-PAGE analysis.

The number of free amino groups was determined by the method of Stocks *et al. (Anal. Biochem.*, 154 :232 (1986)). The PEGylated polypeptides were reacted with 7.5% fluorescamine in 0.1 M sodium phosphate (pH 8.0), and the number of free amino groups was determined by measuring the intensity of fluorescence at 475 nm (excited at 390 nm).

SDS-PAGE analysis was carried out on a 10-20% gradient gel (Daiichi Pure Chemicals Co., Tokyo, Japan). The polypeptides were stained with CBB and the molecular weights were estimated by comparison with standard molecular weight markers (Pharmacia, Upsala, Sweden). Each band was quantified using an image analysis system (Immunomedica model TIF-64). The results are shown in Table 1 below.

Table 1

	Molecular weight distribution (%)					# of
	21K[a]	26K	41K	68K	>91K	free NH_2[b]
Fr45-1				23.0	77.0	6.1
Fr45-2			14.0	52.2	33.8	6.8
Fr45-3		12.0	56.4	28.2	3.4	9.3
Fr45-4	9.9	75.7	14.4			12.6

a: Unmodified hIL-6.

b: Average number of free amino groups per molecule. (cf. hIL-6 has 15 amino groups per molecule.)

EXAMPLE 6

Th four fractions obtained in Example 4 were tested for their capacity to induce IgM production in a B cell leukemic cell line SKW6-C14 (Hirano, T., t *al., Proc. Natl. Acad. Sci. USA*, 82 :5490 (1985)). The PEGylated hIL-6 polypeptides were dissolved in RPMIL-640 medium containing 10% f tal calf serum. Th concentration of th solution was adjusted so that the final volume of the solution added to reaction mixtures (200 µl) was 50 µl. Th results are shown in Tabl 2. The PEGylated hIL-6 retained its activity t induce IgM production in SKW6-C14.

Table 2

	IgM Production (ng/ml)				
	unmodified	PEGylated hIL-6			
Dose(pg/ml)[a]	hIL-6	Fr45-1	Fr45-2	Fr45-3	Fr45-4
3750.0	910	250	335	175	200
937.5	890	120	162	118	175
58.5	545	82	78	70	90
7.5	120	72	73	70	75

a: On the basis of hIL-6 polypeptides.

EXAMPLE 7

The *in vivo* platelet producing activity of the hIL-6, Fr45-0, and Fr100-0, produced in Examples 1 and 3, was examined in mice. Either hIL-6, Fr45-0, Fr100-0, or the mixture of hIL-6 and PEG(4500) (in vehicle) was administered subcutaneously to three Balb/c mice (8 weeks old, female) at a dose of 10 μg/mouse (on the basis of polypeptide) once a day for five days. Control mice were administered only vehicle comprising PBS containing 0.1 % normal mouse serum. Blood samples were taken on the sixth day and platelets in peripheral blood were counted. The results are shown in Table 3.

Fr45-0 and Fr100-0 induced an approximately 230% increase in the number of platelets produced, while unmodified hIL-6, or the mixture of hIL-6 and PEG(4500) induced only an approximately 150% increase.

Table 3

	Number of platelets[a]
Vehicle	74.5 (100%)
PEG(4500)IL-6	173.5 (233%)
PEG(10000)IL-6	170.0 (228%)
hIL-6 + PEG(4500)	117.9 (158%)
hIL-6	113.5 (152%)

a: $\times 10^4/\mu l$, numbers in parentheses are % of the vehicle control.

EXAMPLE 8

The hIL-6 or its PEGylated derivatives (Fr45-1 to Fr45-4), produced in Example 4, were administered subcutaneously to four Balb/c mice (8 weeks old, female) once a day for five days. Control mice were administered only vehicle comprising PBS containing 0.1 % normal mouse serum. Blood samples were taken on the sixth day and platelets in peripheral blood were counted. The results are shown in Table 4. The results also demonstrate a positiv dos response relationship.

The PEGylated hIL-6 showed significantly higher platelet producing activity compared t th unmodified hIL-6.

Table 4

	Number of platelets (% of control)				
		PEGylated hIL-6			
Dose[a]	unmodified hIL-6	Fr45-1	Fr45-2	Fr45-3	Fr45-4
10	126.6**	250.9***	214.4*	194.0**	173.5**
5	130.2*	224.6***	188.9***	188.9***	144.2*
1	111.1	143.6*	151.5*	128.8*	124.0
0.5	115.8	135.4**	125.8**	134.4**	107.2
0.1	100.4	103.2	102.0	92.8	107.6

a: µg/mouse/injection on the basis of hIL-6 polypeptides.
*: P<0.05, **: P<0.01, ***: P<0.001 significant difference from the control (Student's T-test).

EXAMPLE 9

The hIL-6 or its PEGylated derivative (Fr45-2), produced in Example 4, was administered subcutaneously to four acutely thrombocytopenic mice once a day at a dose of 5 µg/mouse for 10 serial days following X-ray irradiation (600 rad). Blood samples were taken daily and platelets in peripheral blood were counted. The results are shown in Figure 1.

PEGylated hIL-6 administration resulted in at least five days earlier recovery from the thrombocytopenia than the control vehicle administration, while unmodified hIL-6 administration resulted in one to two days earlier recovery.

In another test, five X-ray irradiated mice were administered hIL-6 or the Fr45-2 once a day for 7 serial days at a dose of 5 or 50 µg/mouse as described above. Blood samples were taken on the eighth day when the acute decrease of platelets in vehicle administered mice reached the minimum, as is seen in Figure 1, and platelets in peripheral blood were counted. The results are shown in Table 5.

The 5 µg/mouse administration of the Fr45-2 completely prevented the acute drop in the platelet number while even 50 µg/mouse administration of the unmodified hIL-6 did not result in significant recovery from the acute thrombocytopenia.

Table 5

	Number of platelets[a]	
Vehicle	40.8	(63%)
Fr45-2 (5 µg)	83.0	(127%)
hIL-6 (5 µg)	47.0	(72%)
hIL-6 (50 µg)	53.1	(82%)
Normal[b]	65.1	(100%)

a: $\times 10^4/\mu l$, numbers in parentheses are % of the non-irradiated control.
b: Not irradiated with X-ray.

EXAMPLE 10

The hIL-6 or its PEGylated derivative (Fr45-2), produced in Example 4, was administered subcutan ously to four acute-thrombocytopenic mice, that had been treated with 200 mg/kg of a chemical carcinostatic cyclophosphamide (CY), once a day at a dose of 5 µg/mouse for hIL-6 and 1 µg/mouse for PEGylated hIL-6 for 7 serial days following the CY administration. Blood samples were taken daily and platelets in peripheral blood were counted. The results are shown in Figure 2.

PEGylated hIL-6 administration resulted in significantly earlier recovery from the thrombocytopenia than the control vehicle administration, while unmodified hIL-6 administration induced platelet increase but not early recovery.

EXAMPLE 11

As described below, the hIL-6 polypeptide produced in Example 4 was coupled to the activated PEG2 of Example 3.

The hIL-6 polypeptide (100 mg) was dissolved in 200 ml of 0.1 M sodium borate buffer (pH 10.0) and 2500 mg of the activated PEG2 was added to the solution in five additions at 30 min intervals at room temperature with stirring. The reaction mixture was concentrated to 8 ml using YM10 ultrafiltration membrane (Amicon, Danvers, MA, USA) to give a PEG(10000)IL-6 and 3.5 ml of the concentrate was applied to a Superdex G200 column (Pharmacia, Upsala, Sweden) equilibrated with PBS. The PEGylated hIL-6 polypeptide was eluted in five fractions (hereafter referred to as Fr100-1, Fr100-2, Fr100-3, Fr100-4, Fr100-5) with PBS. The yield of each fraction was 3.8 mg, 5.9 mg, 5.8 mg, 4.8 mg, and 4.5 mg ; respectively.

The five fractions obtained were characterized by determining the number of free amino acid groups as described in Example 5. The average number of free amino groups per molecule of each fraction was 5.3, 7.4, 8.6, 9.4, and 10.0 ; respectively.

EXAMPLE 12

An activated PEG12M (a succinimidyl succinate derivative of polyethylene glycol, which is methoxypolyethylene glycol succinate with an average molecular weight of 12,000 and coupled to N-hydroxysuccinimide) was obtained from Nippon Oil & Fats Co. (Tokyo, Japan). The hIL-6 polypeptide produced in Example 4 was coupled to this activated PEG12M. The hIL-6 polypeptide (90 mg) was dissolved in 180 ml of 0.1 M sodium borate buffer (pH 8.5) and 1000 mg of the activated PEG12M was added to the solution in three additions at 30 min intervals in an ice bath with stirring. The reaction mixture was concentrated to 6 ml using YM10 ultrafiltration membrane to give a PEG(12000)IL-6 and was applied to a Superdex G200 column equilibrated with PBS. The PEGylated hIL-6 polypeptide was eluted in five fractions (hereafter referred to as Fr120-1, Fr120-2, Fr120-3, Fr120-4, Fr120-5) with PBS. The yield of each fraction was 1.6 mg, 2.8 mg, 3.5 mg, 3.7 mg, and 3.7 mg ; respectively.

The five fractions obtained were characterized by determining the number of free amino acid groups as described in Example 5. The average number of free amino groups per molecule of each fraction was 5.2, 7.6, 8.7, 9.2, and 9.8 ; respectively.

EXAMPLE 13

The hIL-6 produced in Example 4 or its PEGylated derivatives produced in Examples 11 and 12 were administered subcutaneously to four Balb/c mice (8 weeks old, female) once a day for five days. Control mice were administered with the vehicle. Blood samples were taken on the sixth day and platelets in peripheral blood were counted. The results are shown in Tables 6 and 7.

The PEGylated hIL-6 showed significantly higher platelet producing activity compared to the unmodified hIL-6. Generally, the PEGylated hIL-6 derivatives of Example 12 gave higher activity.

Table 6

Number of platelets (% of control)						
	unmodified	PEGylated hIL-6				
Dose[a]	hIL-6	Fr100-1	Fr100-2	Fr100-3	Fr100-4	Fr100-5
10	143.0***	211.1***	250.8***	204.4***	203.2***	202.4**
5	129.4**	nt	205.9***	212.7***	226.5**	225.9**
1	nt	100.0	148.9**	141.1**	142.5*	142.8*
0.5	nt	nt	107.9	113.7	138.7**	111.3

a: µg/mouse/injection on the basis of hIL-6 polypeptides.
nt: not tested.
*: P<0.05, **: P<0.01, ***: P<0.001 significant difference from the control (Student's T-test).

Table 7

Number of platelets (% of control)						
	unmodified	PEGylated hIL-6				
Dose[a]	hIL-6	Fr120-1	Fr120-2	Fr120-3	Fr120-4	Fr120-5
10	126.7*	201.3*	213.2***	221.4***	200.4***	235.9***
5	nt	246.3***	244.3***	243.1***	229.0***	224.8**
1	nt	221.5**	233.2***	242.7***	221.4**	225.4**
0.5	nt	215.5***	227.8***	205.9***	203.4***	191.5***
0.1	nt	141.3**	167.4***	170.1***	161.5***	154.1***

a: µg/mouse/injection on the basis of hIL-6 polypeptides.
nt: not tested.
*: P<0.05, **: P<0.01, ***: P<0.001 significant difference from the control (Student's T-test).

EXAMPLE 14

The PEGylated hIL-6 produced in Example 12 (Fr120-1 to Fr120-5) were administered subcutaneously to five acute-thrombocytopenic mice (8 weeks old, female), once a day for 7 serial days following X-ray irradiation (600 rad). Blood sampl s wer taken on th ighth day and platelets in periph ral blood wer counted. The results are shown in Figure 3.

All the PEGylated hIL-6 fractions induced prominent recovery from the acute thrombocytopenia. At doses of over 0.1 µg/mouse, the administration of any of the five fractions resulted in a significantly (P<0.01) higher number of platelets produced as compared to th control (vehicle administration only). Fr120-1 and Fr120-2 wer chosen for further study.

EXAMPLE 15

The *in vivo* persistence of the PEGylated hIL-6 in blood was investigated. The hIL-6 of Example 4 or its PEGylat d derivatives (Fr45-2, Fr120-1, and Fr120-2, produced in Examples 4 and 12) were administered subcutaneously to Balb/c mice (8 weeks old, female) at a dose of 0.2 μg/mouse (as polypeptide). Blood samples were taken at intervals and the serum concentration of hIL-6 was determined immunochemically using Quantikine hIL-6 (R&D Systems, Minneapolis, MN, USA). The result is shown in Figure 4.

The PEGylated hIL-6 could be immunochemically detected even 24 hours after the administration while the unmodified hIL-6 could not be detected 5 hours after administration. This strongly suggests that the halflife of hIL-6 was increased by PEGylation.

EXAMPLE 16

The hIL-6 polypeptide, with exactly the sequence shown in Example 4, was produced as described in Example 4 but without the cathepsin C treatment. Thus, this IL-6 has a Met-Lys at the amino terminus. Approximately 2.9 g of the hIL-6 polypeptide, which was a single band on SDS-PAGE and had the predicted molecular weight of 21K, was obtained. The N-terminal sequence was confirmed to be Met-Lys-Ala-Pro- with more than 99% purity by direct sequence analysis. This sequence was stable for at least for 4 months when stored at 4°C.

The hIL-6 polypeptide was PEGylated with the activated PEG12M of Example 12 to give a PEG(12000)IL-6 termed PEG(12000)IL-6'. The five fractions obtained were termed as Fr120'-1 to Fr120'-5.

The PEGylated hIL-6 Fr120'-2 was administered to five mice once a day for five days at a dose of 1 μg-/mouse and blood samples were taken on the sixth day as described in Example 13. The number of peripheral platelets in the test mice showed a 285% increase (significant at $P<0.001$) compared to the control (vehicle administered) mice.

EXAMPLE 17

The acute toxicity of PEGylated hIL-6 was investigated. The hIL-6 of Example 4 or its PEGylated derivatives (Fr45-2, Fr100-2, and Fr120-2), produced in Examples 4, 11, and 12, were administered subcutaneously to five Balb/c mice (6 weeks old, male, 21-23 g weight) at a dose of either 1, 5, or 10 mg/kg weight (as polypeptide). None of the mice was observed to die during the ten day period after the administration. Thus the acute LD^{50} for the three PEGylated hIL-6 was over 10 mg/kg weight.

The foregoing examples describe the PEGylation of IL-6 which substantially increases the biological half-life of IL-6 while still retaining the biological activity of IL-6.

While the present invention has been described in terms of specific methods and compositions, it is understood that variations and modifications will occur to those skilled in the art upon consideration of the present invention. For example, it is anticipated that smaller protein fragments and peptides derived from any form of IL-6, whether glycosylated or unglycosylated, and that still retain IL-6 biological activity, would also be effective in their PEGylated forms. It is also anticipated that forms of PEG other than the succinimidyl succinate derivatives of PEG (formula 5), the bis-polyethylene glycol derivatives of cyanuric chloride (formula 7) and the polyoxyethylene diamines (formula 8), such as carbonyl dimidazole, phenylcarbonate, succinimidyl carbonate or maleimide derivatives of PEG and mono-polyethylene glycol derivatives of cyanuric chloride, as well as other polyoxyethylene derivatives such as polyoxyethylene monoamines, will also be effective as PEGylating agents.

Although the preferred forms of PEGylated IL-6 are unglycosylated hIL-6 polypeptides PEGylated at amino groups with polyethylene glycol succinate, it is not intended to preclude other combinations of PEG and IL-6 that are effective in having improved biological halflife and in retaining IL-6 activity.

Further, it is anticipated that the general method of the invention of preparing PEGylated forms of IL-6 can be used to prepare PEGylated forms of other proteins for which it is desired to improve the biological half-life while still retaining and/or enhancing biological activity. Such other proteins include interleukin-2, interleukin-3, G-CSF, adenosine deaminase, asparaginase, urokinase and superoxide dismutase.

Numerous modifications and variations in the invention as described in the above illustrative examples are xpected to occur to those skilled in the art and consequently only such limitations as appear in the appended claims should be placed th reon.

Accordingly, it is intended in the appended claims to cover all such equivalent variations which come within the scope of the invention as claimed.

SEQUENCE LISTINGS

(1) INFORMATION FOR SEQ. ID. NO. 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 185
(B) TYPE: Amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION : SEQ. ID. NO:1:

SEQUENCE ID NO.: 1

```
ALA PRO VAL PRO PRO GLY GLU ASP SER LYS ASP VAL ALA ALA
                 5                  10
PRO HIS ARG GLN PRO LEU THR SER SER GLU ARG ILE ASP LYS
 15                  20                  25
GLN ILE ARG TYR ILE LEU ASP GLY ILE SER ALA LEU ARG LYS
     30                  35                  40
GLU THR CYS ASN LYS SER ASN MET CYS GLU SER SER LYS GLU
         45                  50                  55
ALA LEU ALA GLU ASN ASN LEU ASN LEU PRO LYS MET ALA GLU
             60                  65                  70
LYS ASP GLY CYS PHE GLN SER GLY PHE ASN GLU GLU THR CYS
                 75                  80
LEU VAL LYS ILE ILE THR GLY LEU LEU GLU PHE GLU VAL TYR
 85                  90                  95
LEU GLU TYR LEU GLN ASN ARG PHE GLU SER SER GLU GLU GLN
    100                 105                 110
ALA ARG ALA VAL GLN MET SER THR LYS VAL LEU ILE GLN PHE
        115                 120                 125
LEU GLN LYS LYS ALA LYS ASN LEU ASP ALA ILE THR THR PRO
            130                 135                 140
ASP PRO THR THR ASN ALA SER LEU LEU THR LYS LEU GLN ALA
                145                 150
GLN ASN GLN TRP LEU GLN ASP MET THR THR HIS LEU ILE LEU
155                 160                 165
ARG SER PHE LYS GLU PHE LEU GLN SER SER LEU ARG ALA LEU
    170                 175                 180
ARG GLN MET
        185
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(2) **INFORMATION FOR SEQ. ID. NO. 2**

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 186

(B) TYPE: Amino acid

(C) STRANDEDNESS: N/A

(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION : SEQ. ID. NO. 2:

SEQ ID NO.: 2

```
MET ALA PRO VAL PRO PRO GLY GLU ASP SER LYS ASP VAL ALA
 -1   1               5                   10
ALA PRO HIS ARG GLN PRO LEU THR SER SER GLU ARG ILE ASP
     15                  20                  25
LYS GLN ILE ARG TYR ILE LEU ASP GLY ILE SER ALA LEU ARG
         30                  35                  40
LYS GLU THR CYS ASN LYS SER ASN MET CYS GLU SER SER LYS
             45                  50                  55
GLU ALA LEU ALA GLU ASN ASN LEU ASN LEU PRO LYS MET ALA
                 60                  65
GLU LYS ASP GLY CYS PHE GLN SER GLY PHE ASN GLU GLU THR
 70                  75                  80
CYS LEU VAL LYS ILE ILE THR GLY LEU LEU GLU PHE GLU VAL
     85                  90                  95
TYR LEU GLU TYR LEU GLN ASN ARG PHE GLU SER SER GLU GLU
        100                 105                 110
GLN ALA ARG ALA VAL GLN MET SER THR LYS VAL LEU ILE GLN
            115                 120                 125
PHE LEU GLN LYS LYS ALA LYS ASN LEU ASP ALA ILE THR THR
                130                 135
PRO ASP PRO THR THR ASN ALA SER LEU LEU THR LYS LEU GLN
140                 145                 150
ALA GLN ASN GLN TRP LEU GLN ASP MET THR THR HIS LEU ILE
    155                 160                 165
LEU ARG SER PHE LYS GLU PHE LEU GLN SER SER LEU ARG ALA
        170                 175                 180
LEU ARG GLN MET
            185
```

(3) INFORMATION FOR SEQ. ID. NO. 3

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 187
(B) TYPE: Amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: N/A

(xi) SEQUENCE DESCRIPTION : SEQ. ID. NO. 3:

SEQ ID NO. 3:

```
MET LYS ALA PRO VAL PRO PRO GLY GLU ASP SER LYS ASP VAL
 -2  -1   1                   5                  10
ALA ALA PRO HIS ARG GLN PRO LEU THR SER SER GLU ARG ILE
         15                  20                  25
ASP LYS GLN ILE ARG TYR ILE LEU ASP GLY ILE SER ALA LEU
             30                  35                  40
ARG LYS GLU THR CYS ASN LYS SER ASN MET CYS GLU SER SER
                 45                  50
LYS GLU ALA LEU ALA GLU ASN ASN LEU ASN LEU PRO LYS MET
 55                  60                  65
ALA GLU LYS ASP GLY CYS PHE GLN SER GLY PHE ASN GLU GLU
     70                  75                  80
THR CYS LEU VAL LYS ILE ILE THR GLY LEU LEU GLU PHE GLU
         85                  90                  95
VAL TYR LEU GLU TYR LEU GLN ASN ARG PHE GLU SER SER GLU
            100                 105                 110
GLU GLN ALA ARG ALA VAL GLN MET SER THR LYS VAL LEU ILE
                115                 120
GLN PHE LEU GLN LYS LYS ALA LYS ASN LEU ASP ALA ILE THR
125                 130                 135
THR PRO ASP PRO THR THR ASN ALA SER LEU LEU THR LYS LEU
    140                 145                 150
GLN ALA GLN ASN GLN TRP LEU GLN ASP MET THR THR HIS LEU
        155                 160                 165
ILE LEU ARG SER PHE LYS GLU PHE LEU GLN SER SER LEU ARG
            170                 175                 180
ALA LEU ARG GLN MET
                185
```

Claims

1. PEG-IL-6 having biological activity.

2. PEG-IL-6 according to claim 1 wherein said IL-6 is glycosylated IL-6, unglycosylated IL-6 or a biologically active fragment thereof.

3. PEG-IL-6 according to claim 2 wherein said IL-6 comprises a sequence of amino acids as defined in the Sequence Listing by SEQ ID NO :1, SEQ ID NO :2 or SEQ ID NO :3.

4. PEG-IL-6 according to any one of the preceding claims, wherein said IL-6 is a naturally produced or synthetically produced IL-6.

5. PEG-IL-6 according to claim 4 wherein said naturally produced IL-6 is obtainable as an expression product of a prokaryotic or eukaryotic host cell transformed or transfected with a DNA sequence encoding IL-6 or encoding a biologically active IL-6 fragment.

6. PEG-IL-6 according to claim 5 wherein said host cell is E. coli.

7. PEG-IL-6 according to any one of the preceding claims wherein said PEG is obtainable from activated PEG.

8. PEG-IL-6 according to claim 7 wherein said activated PEG is the succinimidyl succinate derivative of PEG or the bis-PEG derivative of cyanuric chloride.

9. PEG-IL-6 according to claim 7 wherein said activated PEG is activated PEG1, PEG2 or PEG12M.

10. PEG-IL-6 according to any one of the preceding claims wherein the PEG is attached to the IL-6 via one or more amino groups on said IL-6.

11. PEG-IL-6 according to claim 10 wherein at least one hydrogen atom of said amino group on said IL-6 is substituted with said PEG.

12. PEG-IL-6 according to claim 10 wherein at least one amino group on said IL-6 is substituted with said PEG.

13. PEG-IL-6 according to any one of claims 1 to 9, wherein said PEG is attached via one or more carboxyl groups on said IL-6.

14. PEG-IL-6 according to claim 13 wherein at least one carboxyl group on said IL-6 is substituted with said PEG.

15. PEG-IL-6 according to any one of the preceding claims selected from PEG1-IL-6, PEG2-IL-6 and PEG12M-IL-6.

16. PEG-IL-6 according to claim 15, wherein the PEG1-IL-6 is PEG(4500)IL-6, the PEG2-IL-6 is PEG(10000)IL-6, and the PEG12M-IL-6 is PEG(12000)IL-6 or PEG(12000)IL-6′.

17. PEG-IL-6 according to claim 16, wherein the PEG(4500)IL-6 is selected from Fr45-0, Fr45-1, Fr45-2, Fr45-3, Fr45-4 or Fr45-5 ; the PEG(10000)IL-6 is selected from Fr100-0, Fr100-1, Fr100-2, Fr100-3, Fr100-4 or Fr100-5 ; the PEG(12000)IL-6 is selected from Fr120-1, Fr120-2, Fr120-3, Fr120-4 or Fr120-5 ; and the PEG(12000)IL-6′ is selected from Fr120′-1, Fr120′-2, Fr120′-3, Fr120′-4 or Fr120′-5.

18. PEG-IL-6 as claimed in any one of the preceding claims, for use in the treatment of haematopoietic disorders in an organism.

19. PEG-IL-6 according to claim 18 for use in promoting platelet production.

20. A pharmaceutical composition comprising a pharmaceutically acceptable solvent, diluent, adjuvant or carrier and as an active ingredient, a biologically active PEG-IL-6 as claimed in any one of claims 1 to 17.

FIG. 1 Change of platelet counts after X-ray irradiation

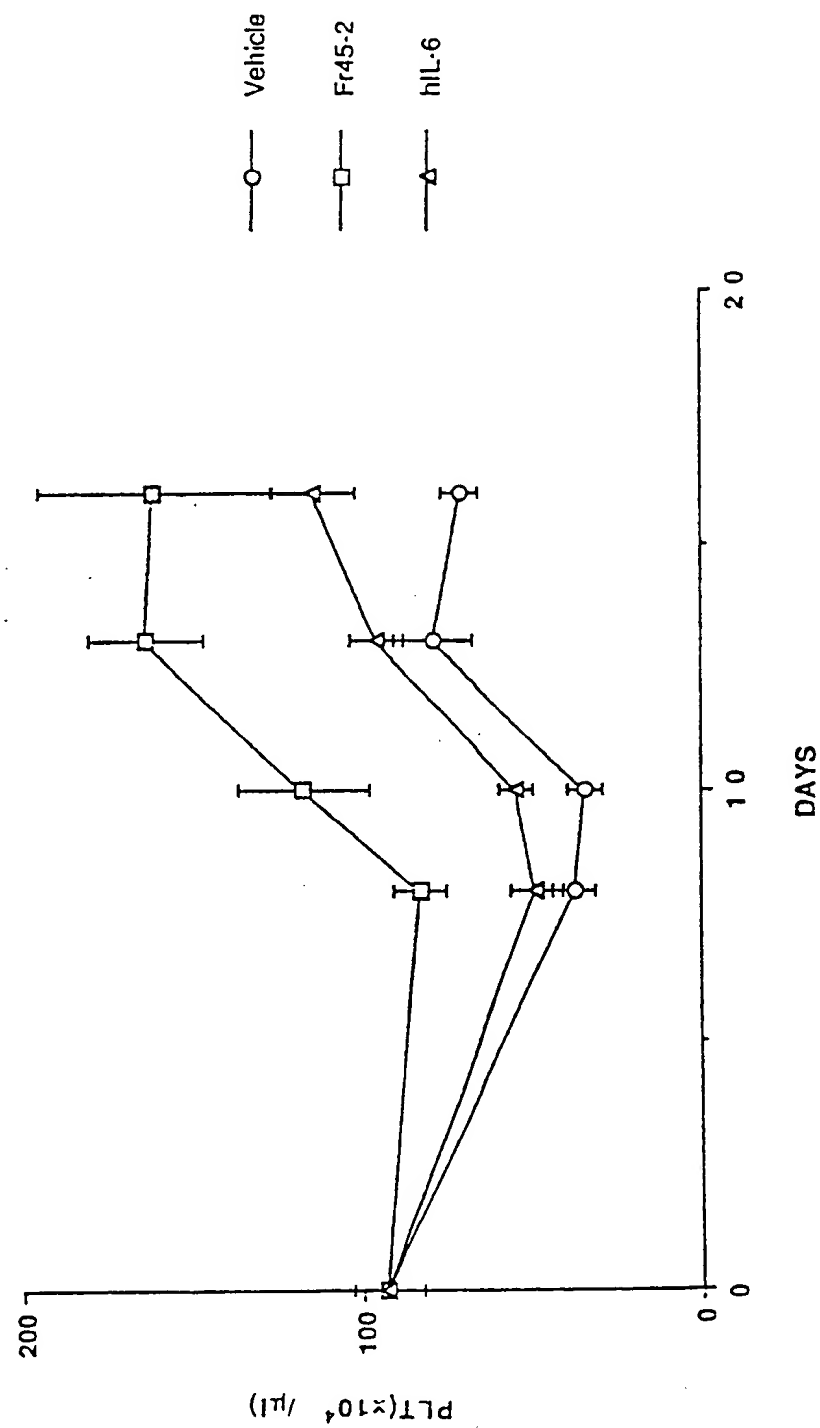


FIG. 2

Change of platelet counts after the administration of Cyclophosphamide

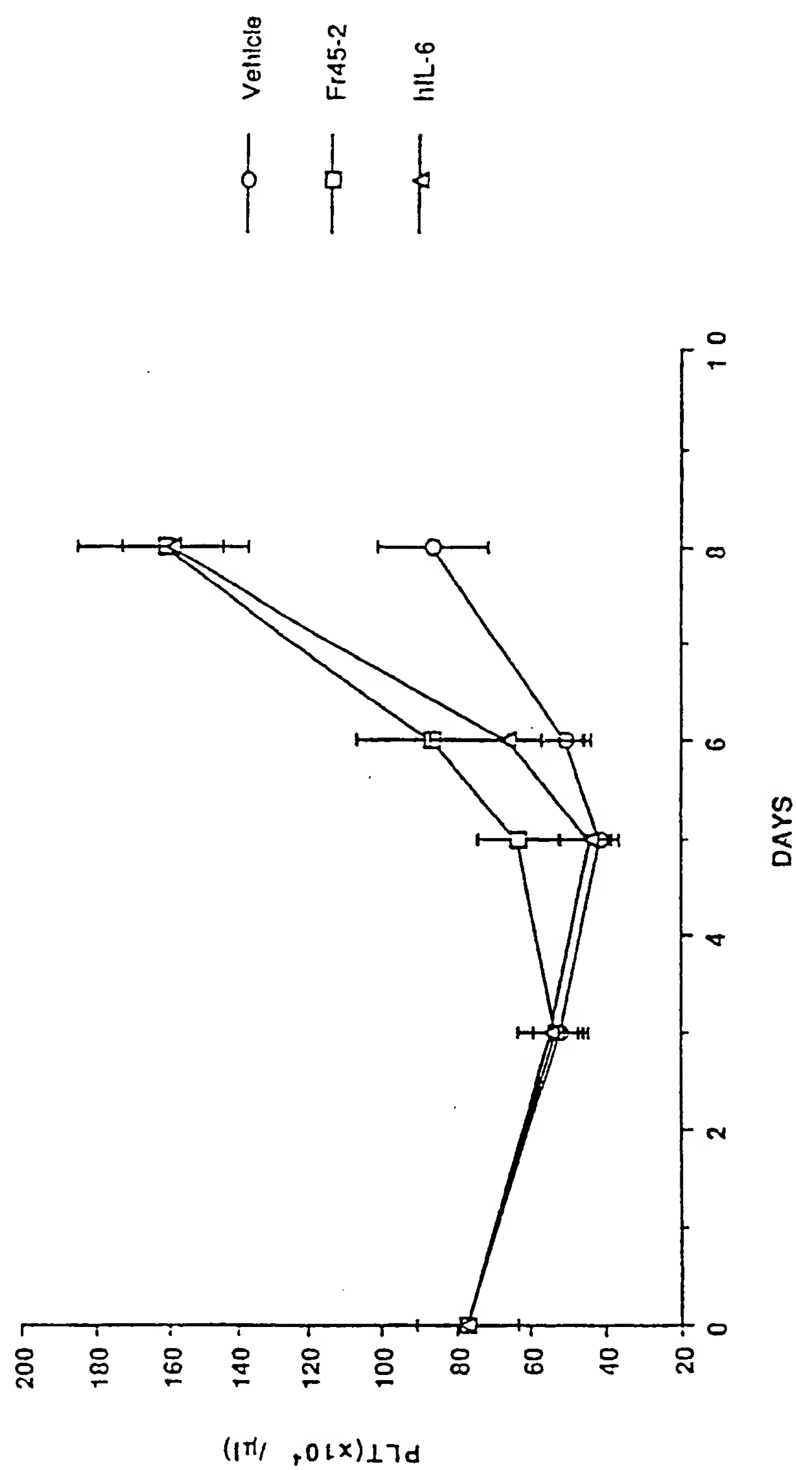
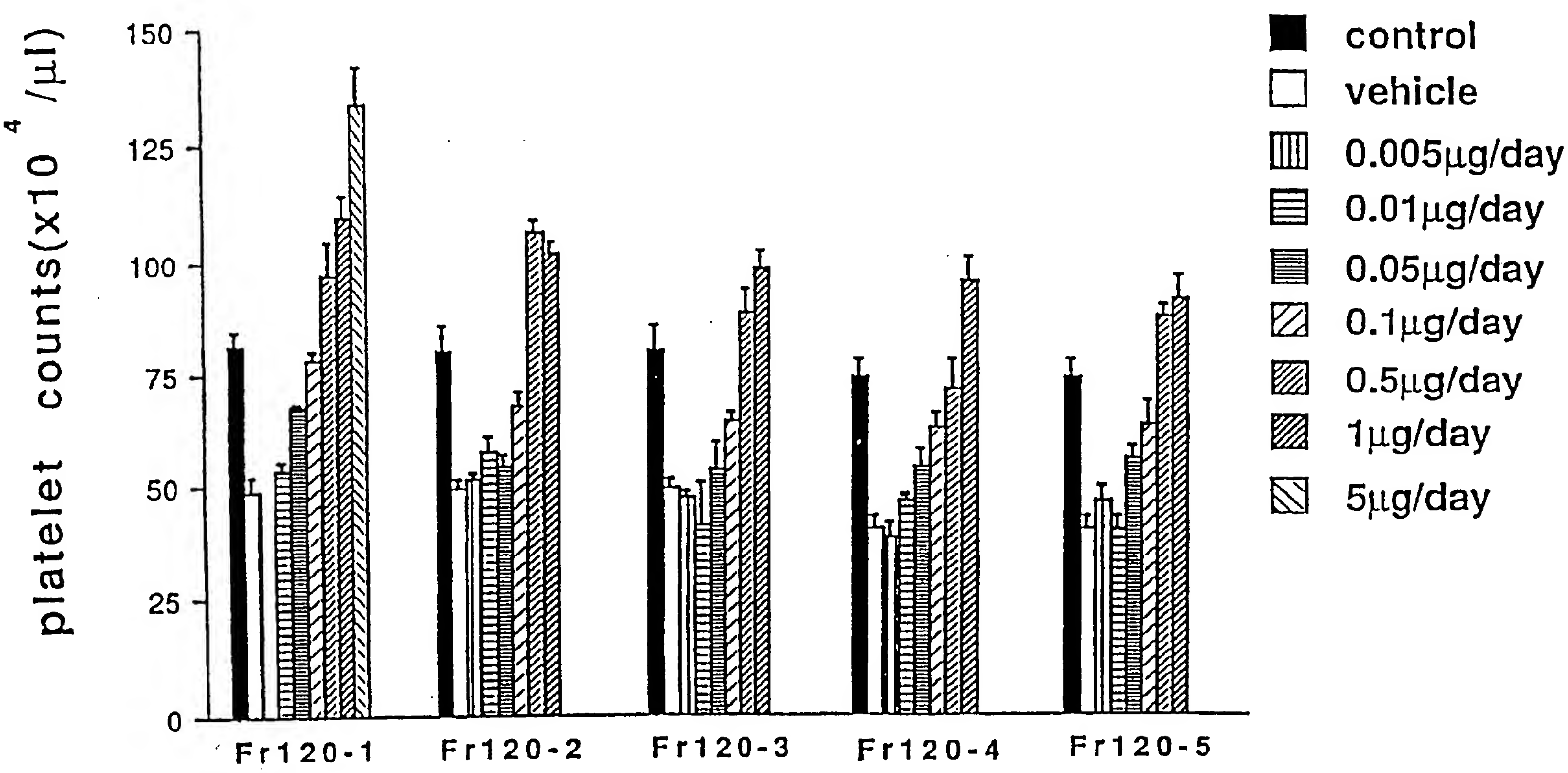


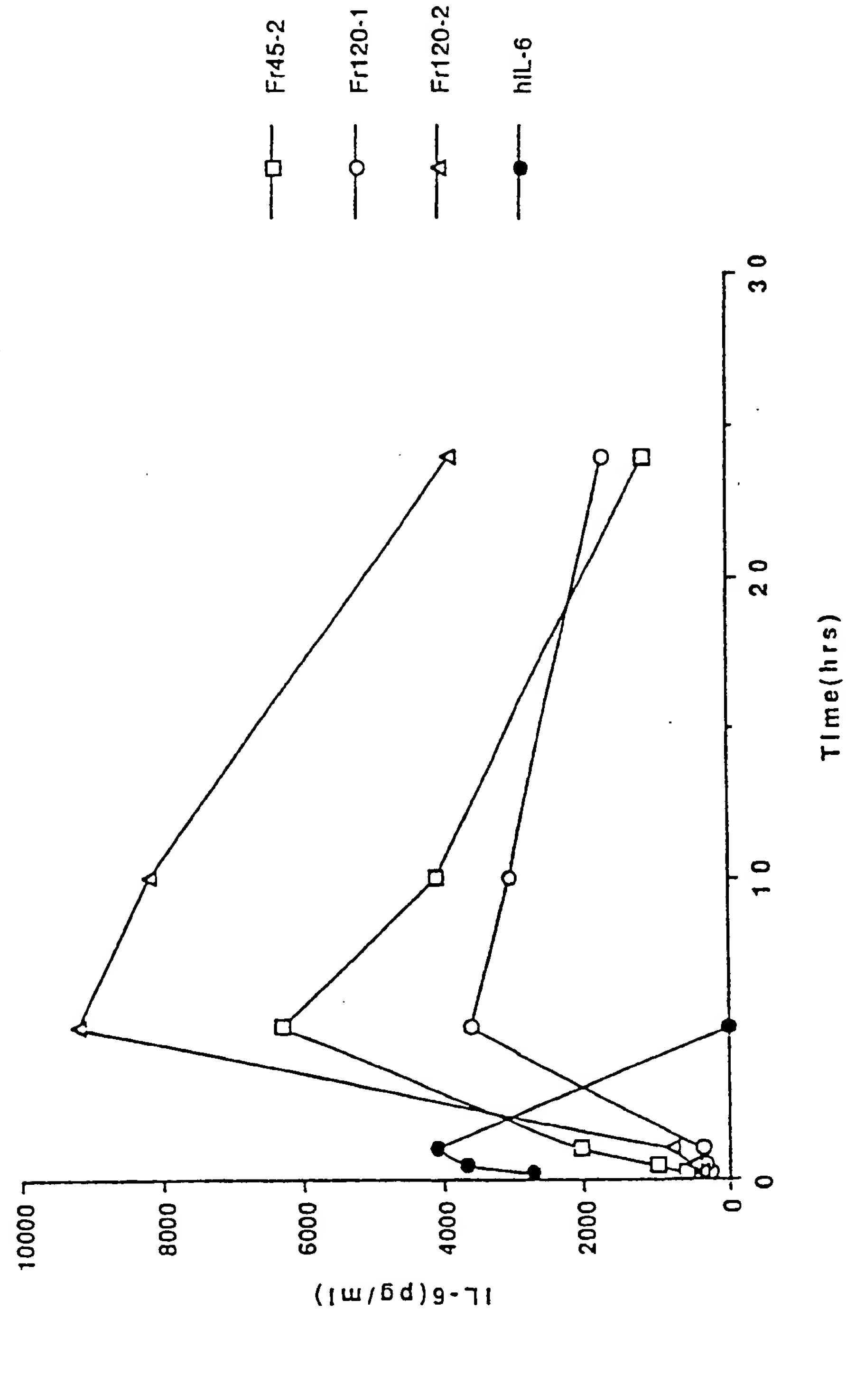
FIG. 3



Effects of PEG/IL-6 on platelet counts in irradiated mice.

FIG. 4

Change of the IL-6 concentration in serum after the administration of PEG/IL-6 or hIL-6(s.c.)



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(54) **Modified hIL-6.**

(57) Provided are PEGylated "interleukin-6" derivatives (PEG-IL-6) having an extended plasma half-life, as well as enhanced *in vivo* IL-6 biological activities.

Methods for producing the modified glycosylated and unglycosylated IL-6 proteins or polypeptides, as well as, for their use in treating hematopoietic disorders and difficiencies, particularly acute thrombocytopenia, are also provided.

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	WO-A-8 905 824 (GENETICS INSTITUTE, INC) * page 3, line 8 - line 12; claims 5,10,12-13; figure 3 *	1-8, 10-12, 18-20	A61K47/48 A61K37/02 C07K13/00 C12P21/02 C12N15/24 C07K17/08
D,P, X	& US-A-4 904 584 (GENETICS INSTITUTE, INC.)		
Y,D	EP-A-0 257 406 (AJINOMOTO CO; INC.) * page 35, line 18 - line 19; claims; examples 9-10 *	1-8, 10-12, 18-20	
Y	EP-A-0 335 423 (KYOWA HAKKO KOGYO CO., LTD.) * page 3, line 10 - page 6, line 50; claims *	1-8, 10-12, 18-20	
D,Y	WO-A-8 700 056 (CETUS CORPORATION) * page 6, line 5 - line 29 * * page 18, line 7 - line 10; examples I-VII * * page 2, line 19 - page 2, line 24 * * page 4, line 24 - page 5, line 13 *	1-8, 10-12, 18-20	TECHNICAL FIELDS SEARCHED (Int. Cl.5) A61K C07K C12N
Y	WO-A-8 604 145 (UNIVERSITY OF NEW MEXICO) * page 11, line 12 - page 12, line 16; claims 1,3,5,13 *	1-8, 10-12, 18-20	

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	18 DECEMBER 1991	LE CORNEC N.D.R.

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	JOURNAL OF CONTROLLED RELEASE vol. 11, no. 1/3, January 1990, AMSTERDAM, NL. pages 139 - 147; F.FUERTGES ET AL: 'The clinical efficacy of polyethylene glycol-modified proteins' * page 140, left column * * page 147, right column, line 27 - line 37 *	1-20	
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